EXTENDED EXPERIMENTAL PROCEDURES

Generation of *Spic*$^{igfp/+}$ Mice

The targeting construct was prepared using the Gateway recombination system (Invitrogen). To construct pENTR loxFRT rNEO IRES-EGFP, a floxed PGK (phosphoglycerate kinase promoter-neomycin phosphotransferase)-neo^R gene cassette (1,982 bp) was excised from the pLNTK targeting vector using SalI and XhoI. After incubation with Easy-A cloning enzyme (Stratagene) and dNTPs to generate 3′-dideoxy adenine overhangs, this PGK–neo^R cassette was ligated into the multiple cloning site of pGEM T-Easy (Promega). The resulting 2,022 bp PGK–neo^R gene cassette was released using NotI and ligated into the 2,554 bp backbone of NotI digested pENTR lox-Puro. Flippase Recognition Target (FRT) sites were sequentially inserted at the SacI and HindIII sites using DNA fragments generated by following annealing oligonucleotides: SacII-FRT-A (GAAGTTCCTATTCCGAAGTTCCTATTCTC TAGAAAGTATAGGAACTTCCGC) and SacII-FRT-B (GGAAGTTCCTATACTTTCTAGAGAATAGGAACTTCGGAATAGGAACTTCGC); or HindIII-FRT-A (AGCTTGAAGTTCCTATTCCGAAGTTCCTATTCTCTAGAAAGTATAGGAACTTC) and HindIII-FRT-B (AGCTGAAG TTCCTATACTTTCTAGAGAATAGGAACTTCGGAATAGGAACTTCA).

IRES2-EGFP was excised from pIRES2-EGFP (BD Biosciences Clontech) using BamHI and AfI II, and blunted using VentR DNA Polymerase (New England BioLabs). This fragment was inserted into the pENTR loxFRT rNEO, which was digested with ScaII and blunted using VentR DNA polymerase. To construct pENTR-5′arm, the 5′arm was generated by PCR from genomic DNA using the following oligonucleotides which contain attP4 and attB1R sites: GGGGACAACTTTGTATAGAAAAGTTGTCTAACTCACAGAA ACCCAGTGTTT, and GGGGACTGCTTTTTTGTACAAACTTGGAATTGAAGAGTCTTGGGTTGCGGA. The attP4-attB1R PCR fragment was ligated into pDONR(P4-P1R) plasmid (Invitrogen) by BP recombination reaction. To construct pENTR-3′arm, the 3′arm was generated by PCR from genomic DNA using the following 12 oligonucleotides which contain attb2 and attB3 sites: GGGGAC AGCTTTCTTGTACAAAGTGGGTCAGTCGGCATTTTTTTAAAAA and GGGGACAACTTTGTATAATAAAGTTGCCTATGCTGGTCTCC CCCACATTTT. The attb2-attB3 PCR fragment was ligated into pDONR (P2-P3) plasmid (Invitrogen) by BP recombination reaction. The LR recombination reaction was performed to generate the final targeting construct by using these three entry clones.

Antibodies

The following antibodies were used for flow cytometry, cell sorting, immunohistochemistry, and western blots as noted. APC anti-F4/80 (MF48005, Invitrogen), Pacific Blue anti-F4/80 (MF48028, Invitrogen), PE-Cy7 anti-CD11b (25-0112-82, eBioscience), eFluor450 anti-MHC-II (48-5321-82), PE anti-TremL4 (143303, BioLegend), eFluor450 anti-VCAM1 (48-1061-80), biotin anti-VCAM1 (13-1061-82, eBioscience), FITC anti-VCAM1 (11-1061-82, eBioscience), PE anti-Ly6G (551461, BD Pharmingen), V450 anti-Ly6C (560594, BD Biosciences), PerCP-Cy5.5 anti-Ter119 (560512, BD Biosciences), biotin anti-Ter119 (13-5921-82, eBioscience), anti-GFP (A11122, Invitrogen), FITC anti-CD45.1 (553775, BD Pharmingen), APC eFluor780 anti-CD45.1 (47-0453-82, eBioscience), PE-Cy7 anti-CD45.1 (25-0453-82, eBioscience), PerCP-Cy5.5 anti-CD45.1 (45-0453-82, eBioscience), APC eFluor780 anti-CD45.2 (47-0454-82, eBioscience), PerCP-Cy5.5 anti-CD45.2 (45-0454-80, eBioscience), eFluor450 anti-CD45R (48-0452-82, eBioscience), APC anti-CD3e (553066, BD Pharmingen), APC anti-human CD4 (MHCD0427, Invitrogen or 300514, BioLegend), PE anti-human CD4 (MHCD0404, Invitrogen), anti-Bach1 (AF5777, R&D Systems), anti-tubulin (53463, Cell Signaling), HRP anti-goat, biotin anti-CD68 (ABD Serotec), APC anti-CD169 (ABD Serotec), 488 anti-goat IgG (A11055, Invitrogen), Alexa Fluor 647 streptavidin (1024071, Invitrogen) and PE-Cy7 streptavidin (557598, BD Pharmingen). 7-AAD (51-6898, BD Biosciences) was used to label live cells for flow cytometry-based studies. DAPI (1140028, Invitrogen) was used to label nuclei on frozen sections.

Chemicals and Drugs

The following chemicals were used for in vitro and in vivo experiments as indicated: heme (Hemin, 51280-1G, Sigma), bilirubin (B584-9, Frontier Scientific), biliverdin (Biliverdin hydrochloride, B655-9, Frontier Scientific), chromium mesoporphyrin (Cr(III) Mesoporphyrin(IX) Chloride, CrM459, Frontier Scientific), phenylhydrazine (Phenylhydrazine hydrochloride, 114715-5G, Sigma), Nr1d1 agonist (GSK4112, Tocris Biosciences), LXR agonist (GW3965, Sigma), Ppar-γ agonist (Pioglitazone hydrochloride, 4124, Tocris Bioscience), RXR agonist (Retinoic acid, R2625, Sigma), and deferoxamine (Deferoxamine mesylate salt, D9533-1G, Sigma). The Hemin stock solution was prepared at 25 mg/ml in 0.15 M NaCl containing 10% NH_4OH (vehicle) and stored at $-20^{\circ}C$. The phenylhydrazine solution was prepared fresh at 10 mg/ml in 1X PBS (sterile, pH 7.4). The chromium mesoporphyrin stock solution was prepared at 75 mM concentration in 50 mM Na_2CO_3 solution. For intraperitoneal treatment, stock Crmp was diluted in sterile 1X PBS prior to injections. Cycloheximide (Sigma-Aldrich). MG132 (Sigma-Aldrich). OCT compound (4583, Tissue-Tek). CAS blocking reagent (008120, Invitrogen).

Cloning and Retrovirus Infection

The cDNAs used in this study were amplified from mouse splenocyte RNA using Superscript III (Invitrogen). The cDNAs and the corresponding primers are as follows: *Spic* cDNA was amplified with forward primer 5′-AGAGATCTGCAACCCAAGACTCTTCAATTC-3′ and reverse primer 5′-CCACTCGAGAGGCAAGAAGCTGGGGTCA-3′; *Tcfec* cDNA was amplified with forward primer 5′-CCGGA GTTCTATGAGCAAGC-3′ and reverse primer 5′-TGCGGCTTACAACTCATCAC-3′; *Bach1* cDNA was amplified with forward primer 5′-TGTGATCACGGTCGATGACAGTGAGAAGCATG-3′ and reverse primer 5′ – TAGTCGACTGCCCGTGGGGTTTACTCGT – 3′. Aforementioned cDNAs were cloned into BglII and XhoI digested GFP-RV retroviral constructs to generate the respective retroviral

overexpression vectors that were next transfected into Phoenix-E cells as described previously (Kanbe and Zhang, 2004; Sedy et al., 2005), and viral supernatants were collected 2 days later. BM cells were infected with viral supernatants in the presence of 2 μg/ml polybrene by spin infection at 2,500 rpm for 45 min.

Quantitative Real-Time PCR

Total RNA and cDNA were prepared with the RNeasy Mini Kit (QIAGEN) and Superscript III reverse transcriptase (Invitrogen). Real-time PCR analysis was performed using ABI SYBR Green master mix and StepOnePlus Real-Time PCR system (Applied Biosystems).The PCR conditions were 10 min at 95°C followed by 40 two-step cycles consisting of 15 s at 95°C and 1 min at 60°C. Primer sequences used to evaluate relative gene expression were followings: *Spic* forward, 5′ – TCCGCAACCCAAGACTCTTCAA – 3′; *Spic* reverse, 5′ – GGGTTCTCTGTGGGTGACATTCCAT – 3′; HPRT forward, 5′-TCAGTCAACGGGGGACATAAA-3′; and HPRT reverse, 5′-GGGGCTGTACTGCTTAACCAG-3′.

Cell Preparation

BM cells were harvested by flushing the femur and tibia with Iscove's modified Dulbecco's medium (IMDM) with 10% FCS. Peritoneal cells were harvested by injecting and collecting IMDM + 10% FCS into the peritoneal cavity of euthanized mice. Solid organs were harvested and digested in 0.25 mg/ml collagenase B (Roche) and 30 U/ml DNase I (Sigma-Aldrich) for 30 min with constant agitation at 37°C. Cells collected from the BM, peritoneum, and solid organs were treated with ACK lysis buffer to remove erythrocytes, filtered through 80 μm filter, and resuspended in appropriate buffer.

Flow Cytometry and Cell Sorting

For flow cytometry or cell sorting, single-cell suspension in MACS buffer (0.5% BSA and 2 mM EDTA in PBS at pH 7.2) were blocked with anti-CD16/32 (clone 2.4G2; BD) for 5-10 min at 4°C followed by labeling with the indicated antibodies. All flow cytometry data were collected on a FACS Canto (BD Biosciences) and analyzed with FlowJo software (Tree Star). Cell sorting (post-sort purity > 90%) was carried out using the FACSAria II (BD Biosciences). To obtain purified *Spic*-EGFP^{-} and *Spic*-EGFP^{+} monocytes for microarray analysis, spleens were prepared from *Spic*^{igfp} mice as described above and were first negatively selected for CD4, CD8, and B220 by MACS (Miltenyi Biotech) purification using the respective microbeads. Negatively selected splenocytes were then stained with anti-CD11b and anti-Ly6C and sorted. Purified RPM were obtained by staining splenocytes with anti-F4/80 and sorting for F4/80^{hi} *Spic*-EGFP^{hi} cells.

Microarray Analysis

For expression analysis of splenic monocytes, pre-RPM, and RPM, total RNA was isolated from cells using RNeasy Kit (QIAGEN). Biotinylated antisense cRNA was generated using a two-cycle target preparation kit (Affymetrix). After fragmentation, cRNA was hybridized to Affymetrix Mouse Genome 430 2.0 arrays. Data were normalized and expression values were modeled using ArrayStar 4 (DNASTAR).

For expression analysis of heme and vehicle exposed GM-CSF cultures of BM cells (WT, *Spic*$^{igfp/igfp}$, and *Bach1*$^{-/-}$), total RNA was isolated from cells using RNeasy Kit (QIAGEN). RNA was amplified, labeled, and fragmented using the NuGEN PicoSL system and hybridized to the Affymetrix Mouse Gene 1.0 ST arrays. Data were normalized and expression values were modeled using ArrayStar 4 (DNASTAR).

Histology

Tissue was fixed in 4% buffered formalin, cryoprotected with 30% sucrose, and frozen in OCT compound. 6-8 μm sections of the frozen tissue were prepared and blocked with the CAS blocking reagent. Sections were then stained with indicated antibodies following standard immunohistochemistry protocols. Epifluoroscence microscopy was performed using an AxioCam MRn microscope equipped with an AX10 camera (Carl Zeiss). Monochrome images were acquired with AxioVision software (Carl Zeiss) and subsequent color balancing and overlaying was performed with ImageJ software (National Institutes of Health).

SUPPLEMENTAL REFERENCES

Kanbe, E., and Zhang, D.E. (2004). A simple and quick method to concentrate MSCV retrovirus. Blood Cells Mol. Dis. *33*, 64–67.

Sedy, J.R., Gavrieli, M., Potter, K.G., Hurchla, M.A., Lindsley, R.C., Hildner, K., Scheu, S., Pfeffer, K., Ware, C.F., Murphy, T.L., and Murphy, K.M. (2004). B and T lymphocyte attenuator regulates T cell activation through interaction with herpesvirus entry mediator. Nat. Immunol. *6*, 90–98.

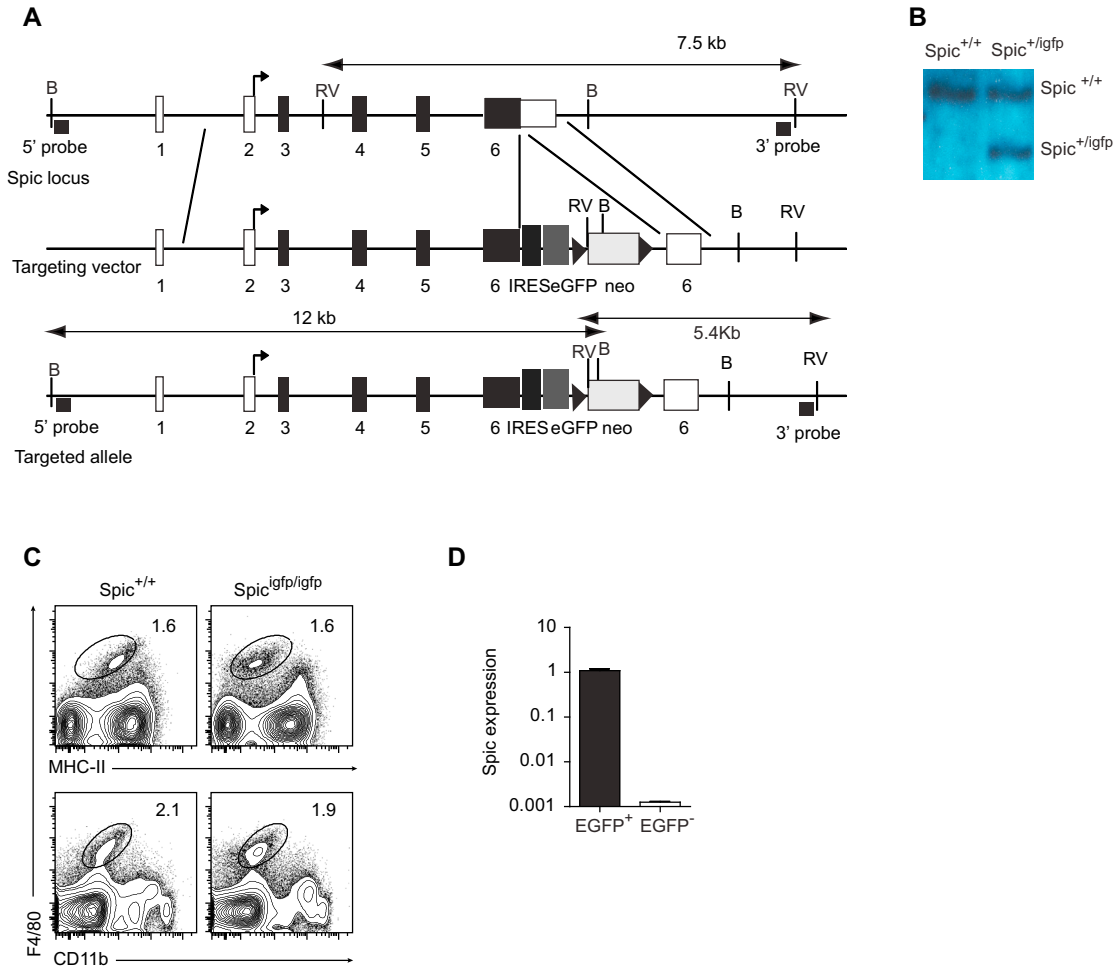

Figure S1. Generation of *Spic*$^{igfp/+}$ Mice, Related to Figure 1

(A) The endogenous *Spic* locus (top), *Spic-Ires-Egfp* targeting vector (middle), and the correctly targeted *Spic* loci (bottom) are shown. Coding (black) and noncoding (open) exons are represented as numbered boxes. BamHI digestion of the endogenous locus generates a restriction fragment of 11 kb detected by the 5′probe, and EcoRV digestion of the endogenous locus generates a restriction fragment of 7.5 kb detected by the 5′probe and 3′probe. In clones retaining the neomycin resistance cassette, the 5′ probe detects a 12 kb fragment, and the 3′probe detects a 5.4 kb fragment. B, BamHI; RV, EcoRV; neo, neomycin resistance cassette.

(B) Shown is a Southern analysis using 3′ probe shown in (A) that hybridizes to EcoRV-digested genomic DNA from *Spic*$^{+/igfp}$ mice or *Spic*$^{+/+}$ mice.

(C) Splenocytes from *Spic*$^{+/+}$ and *Spic*$^{igfp/igfp}$ were stained with F4/80, MHC-II, and CD11b. Shown are the flow cytometry plots with indicated markers. Numbers represent percentage of cells within indicated gate. Data represent more than 3 experiments with 2 or more mice per group.

(D) Shown is the quantitative real-time PCR analysis of *Spic* transcripts isolated from EGFP^{+} and EGFP^{-} splenocytes purified by cell sorting from *Spic*$^{igfp/+}$ mice. Expression was normalized to *Hprt* expression and is presented relative to the expression in EGFP^{+} cells. Data are combined from three independent experiments.

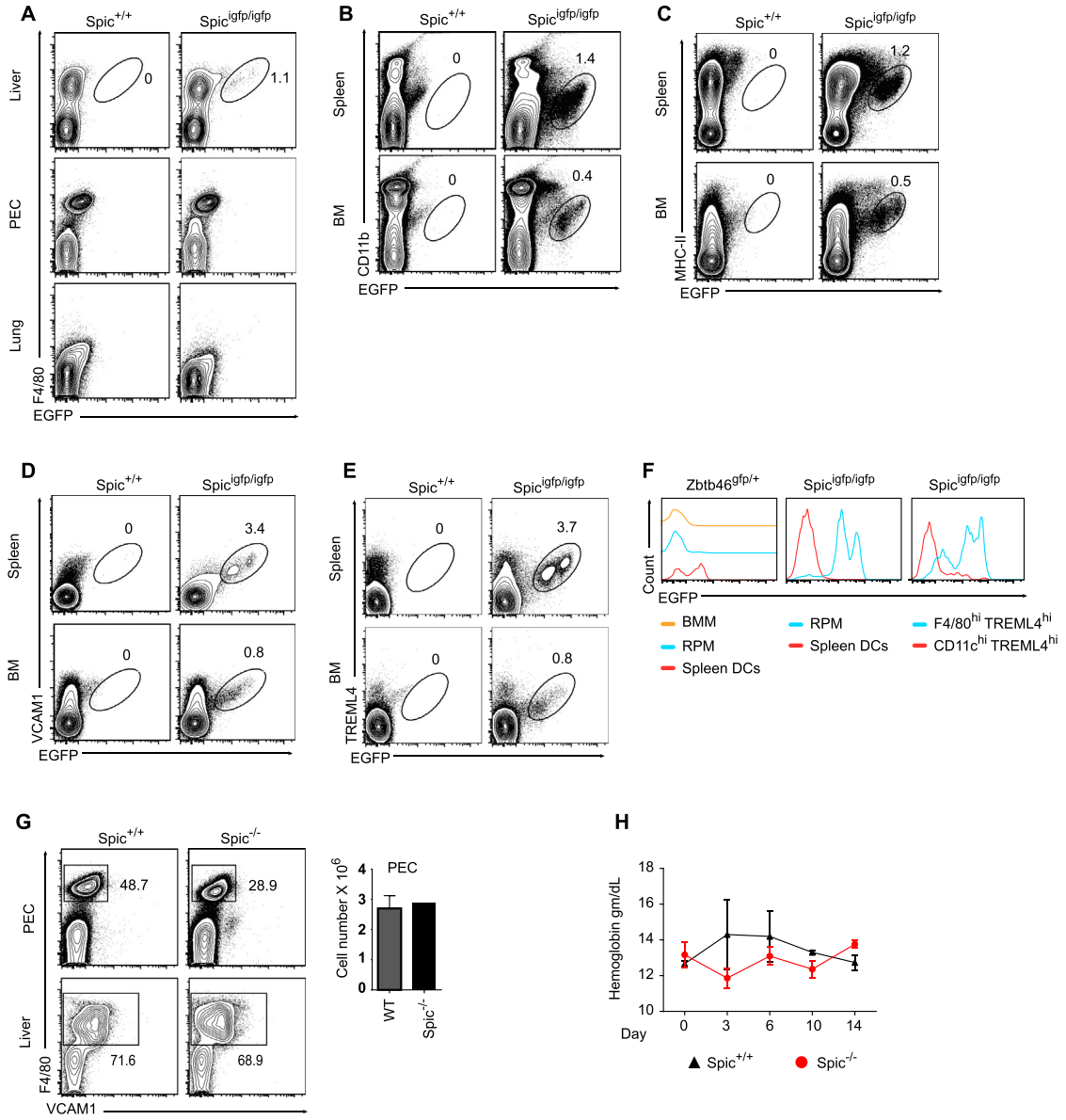

Figure S2. *Spic* Expression Is Specific to RPM and BMM and Is Required for Their Development, Related to Figure 1

(A) Cells from liver (top), peritoneal cavity (middle), and lung (bottom) were harvested from *Spic*$^{+/+}$ and *Spic*$^{igfp/igfp}$ mice and stained for CD45 and F4/80. Shown are the flow cytometry plots for the indicated markers after gating for CD45 expression.

(B–E) BM cells and splenocytes from *Spic*$^{+/+}$ and *Spic*$^{igfp/igfp}$ were stained for CD11b (B), MHC-II (C), VCAM1 (D), and TREML4 (E), as indicated in the flow cytometry plots.

(F, left) Shown is the single-color histogram for *Zbtb46*-EGFP expression in CD11c^{hi} dendritic cells and F4/80^{hi} BMM and RPM from *Zbtb46*$^{gfp/+}$ mice. (F, middle) Shown is the single-color histogram for *Spic*-EGFP expression in splenic RPM (F4/80^{hi}CD11b^{lo}) and splenic dendritic cells (CD11c^{hi} MHC-II^{hi}). (F, right) shown is the single-color histograms for *Spic*-EGFP expression in TREML4-expressing splenic macrophages and TREML4-expressing splenic dendritic cells.

(G) Cells from the peritoneal cavity (top) and liver (bottom) were collected from *Spic*$^{+/+}$ and *Spic*$^{-/-}$ mice and stained for F4/80 and VCAM1. Shown are the flow cytometry plots for the indicated markers. Right: absolute number of F4/80^{hi} peritoneal macrophages (bars: mean + SD, n = 3). Numbers in the flow cytometry plots represent percentage of cells within indicated gate.

(H) Blood was collected by tail bleeding in EDTA tubes (Microvette, Sarstedt) on the indicated days (x axis) after treatment with 200 μl of PBS or PDZ (2 mg in PBS) on day 0. CBC was performed and the hemoglobin concentration (gram/deciliter) is plotted on the y axis. Differences in hemoglobin levels are not significant ($p > 0.05$, unpaired t test) at any time point. All data in this figure are representative of two or more experiments with at least two mice per group. PEC, peritoneal cells.

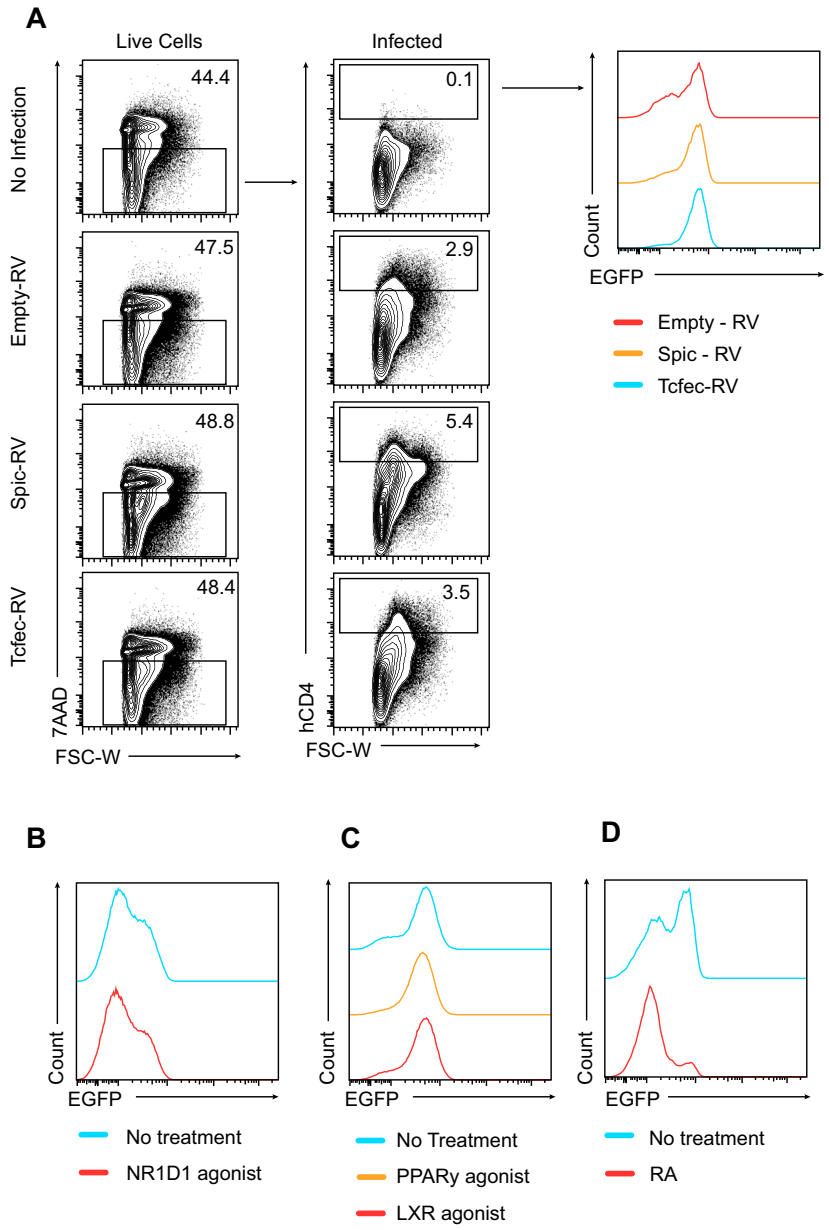

Figure S3. RPM-Associated Transcription Factors Do Not Regulate *Spic* Expression, Related to Figure 2

(A) GM-CSF cultures of *Spic*$^{igfp/igfp}$ BM cells were infected with retroviral vectors expressing only human *Cd4* (Empty-RV), expressing *Spic* and human *Cd4* (*Spic*-RV), and expressing the transcription factor *Tcfec* and human *Cd4* (Tcfec-RV). Seven days after infection, cells were harvested and stained for 7AAD and hCD4. Shown are the flow cytometry plots with the indicated markers. 7AAD$^-$ live cells (column 1) gated for human CD4 expression (column 2) represent live cells infected with the respective retrovirus. These 7AAD$^-$hCD4$^+$ cells were then analyzed for EGFP expression as shown in the single-color histogram on right. Numbers in the flow cytometry plots represent percentage of cells within indicated gate.

(B) GM-CSF cultures of *Spic*$^{igfp/igfp}$ BM cells were treated with the Nr1d1 agonist GSK4112 (10 μM). Cells were collected after 8 days and analyzed for EGFP expression after gating out autofluorescence as shown is the single-color histogram.

(C) GM-CSF cultures of *Spic*$^{igfp/igfp}$ BM cells were treated with LXR agonist GW3965 hydrochloride (1 μM), Ppar-γ agonist pioglitazone hydrochloride (1 μM), or vehicle after 4 days in culture. Cells were collected 5 days after treatment and analyzed for EGFP as shown in the single-color histogram.

(D) GM-CSF cultures of *Spic*$^{igfp/igfp}$ BM cells were treated with RXR agonist retinoic acid (10 μM). Cells were harvested after 10 days and analyzed for EGFP as shown in the single-color histogram.

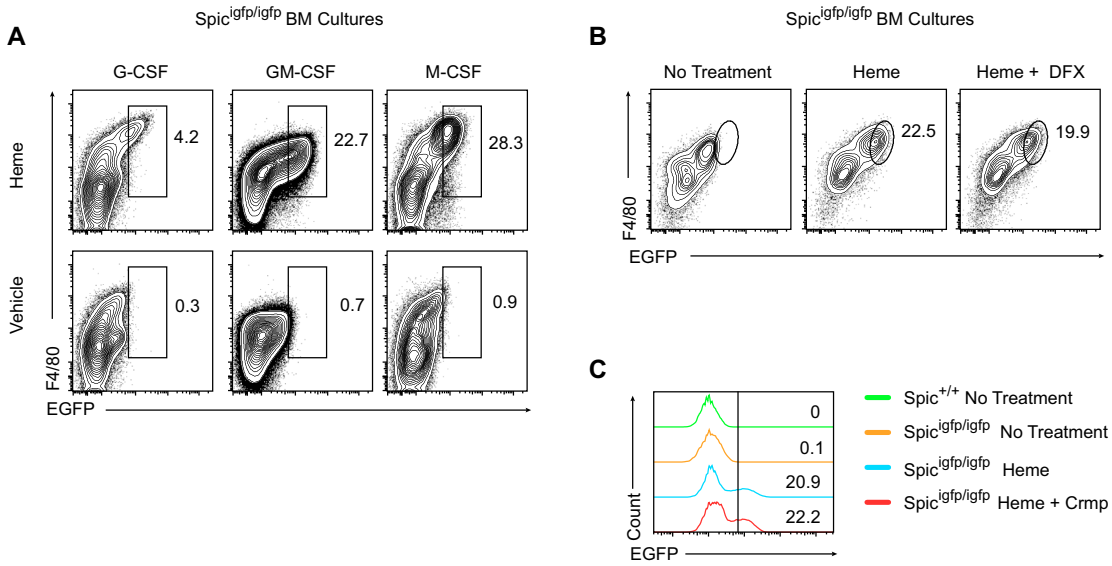

Figure S4. Heme, but Not Heme Metabolites, Induces *Spic* Expression in Macrophages Generated In Vitro, Related to Figure 2

(A) BM cells from *Spic*$^{igfp/igfp}$ were cultured in 20 ng/ml of GM-CSF, G-CSF, or M-CSF. After 5 days, heme (40 μM) or vehicle was added to the culture. Cells were harvested 2 days after treatment and stained with F4/80. Shown are flow cytometry plots with the indicated markers.

(B) GM-CSF cultured *Spic*$^{igfp/igfp}$ BM cells were treated with heme (40 μM) with or without the iron chelator deferoxamine (20 μM) at the onset of culture. Cells were harvested after 10 days and stained with F4/80. Shown are the flow cytometry plots with the indicated markers.

(C) GM-CSF cultured *Spic*$^{igfp/igfp}$ BM cells were treated with heme (30 μM) with or without the HO1 inhibitor Crmp (10 μM) at the onset of culture. Eight days later, the cells were collected and analyzed for EGFP expression. Numbers in the flow cytometry plots represent percentage of cells within the indicated gate. Data in this figure are representative of two or more experiments with at least two mice or samples in each group.

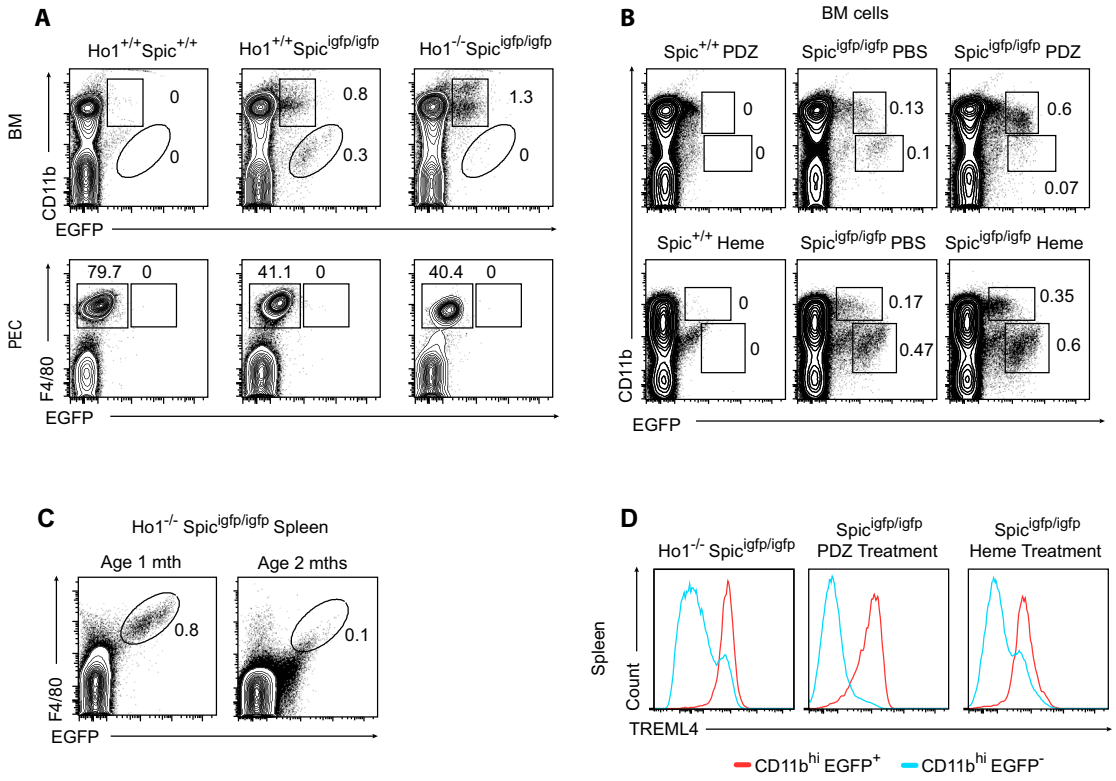

Figure S5. Heme Induces Pre-RPM In Vivo, Related to Figure 3

(A) Cells from the BM (top) and peritoneal cavity (bottom) were harvested from WT, *Spic*$^{igfp/igfp}$, and *Ho1*$^{-/-}$*Spic*$^{igfp/igfp}$ mice and stained with F4/80 and CD11b. Shown are the flow cytometry plots with the indicated markers.

(B, top) *Spic*$^{igfp/igfp}$ and WT mice were treated with 200 μl intraperitoneal PBS or PDZ (2 mg in PBS) three times, each treatment 48 hr apart. 12 hr after the last treatment, BM cells were harvested and stained for CD11b as shown in the flow cytometry plots. (B, bottom) Wild-type and *Spic*$^{igfp/igfp}$ mice were treated intraperitoneally with 200 μl of PBS or heme (0.5 mg in PBS) on three consecutive days. 24 hr after the last treatment, BM cells were harvested and stained for CD11b. Shown are the flow cytometry plots with the indicated markers.

(C) Splenocytes were harvested from 1-month-old and 2-month-old *Ho1*$^{-/-}$*Spic*$^{igfp/igfp}$ mice. Cells were stained with F4/80. Shown is the flow cytometry plot with the indicated markers.

(D) Splenocytes from *Ho1*$^{-/-}$ *Spic*$^{igfp/igfp}$ mice (left), *Spic*$^{igfp/igfp}$ mice treated with PDZ (middle), and *Spic*$^{igfp/igfp}$ mice treated with heme (right) were stained for CD11b and TREML4. Shown are the flow cytometry plots displaying TREML4 expression on CD11b^{hi}EGFP^{+} (red) and CD11b^{hi}EGFP^{-} (blue) cells. Numbers in the flow cytometry plots represent percentage of cells within the indicated gate. All data in this figure is representative of two or more experiments with at least two mice or samples per group.

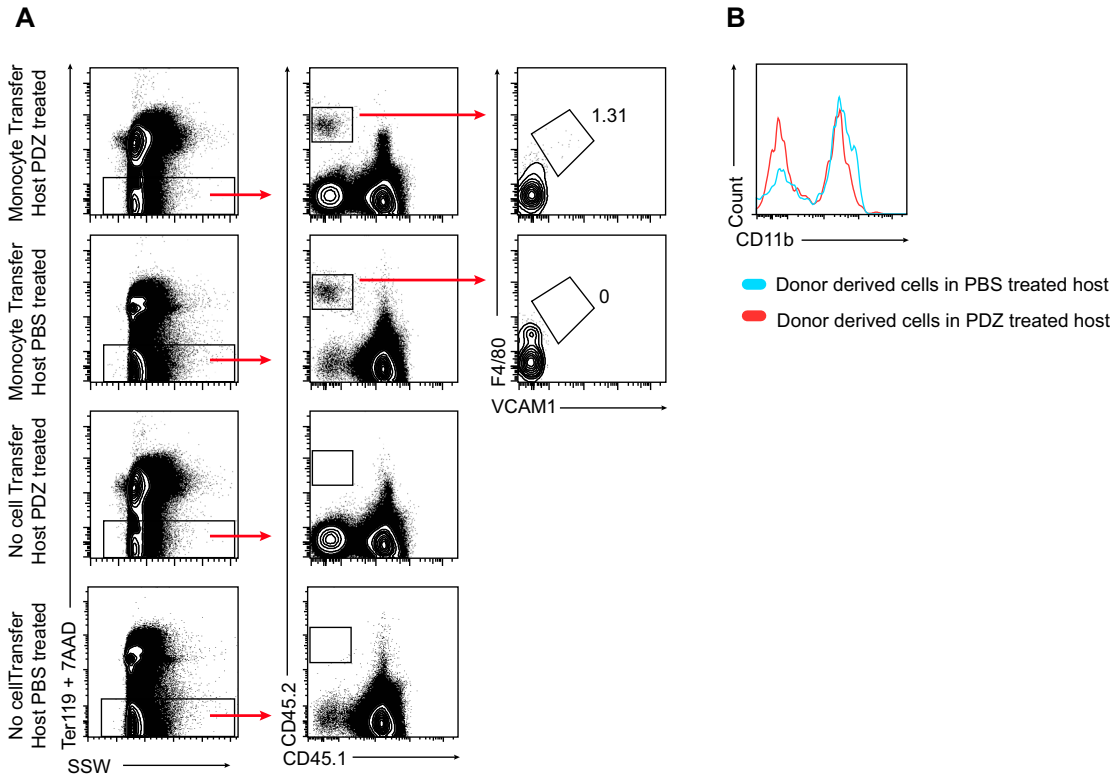

Figure S6. Monocytes Give Rise to Pre-RPM and RPM in the Presence of Heme, Related to Figure 4

(A) Monocytes were purified by MACS-based depletion of $CD3^+$, $B220^+$, and $Ly6G^+$ cells followed by MACS-based enrichment for $CD11b^+$ cells from $CD45.2^+$ C57BL/6 splenocytes. Recipient $CD45.1^+$ C57BL/6 mice were conditioned with PDZ or PBS treatment prior to transfer with about 6×10^6 purified monocytes via tail vein injection. Recipient splenocytes were harvested 4 days after monocyte transfer and stained for Ter119, 7AAD (vital dye), CD45.2, CD45.1, F4/80, VCAM1, and CD11b. Shown are the flow cytometry plots with the indicated markers demonstrating the presence of $F4/80^{hi}VCAM^{hi}$ RPM-like donor cells selectively among splenocytes of hosts experiencing hemolysis (PDZ treated).

(B) Shown is the single-color histogram displaying the expression of CD11b in donor-derived monocytes in the presence (red) or absence (blue) of hemolysis. Numbers in the flow cytometry plots represent percentage of cells within indicated gate.

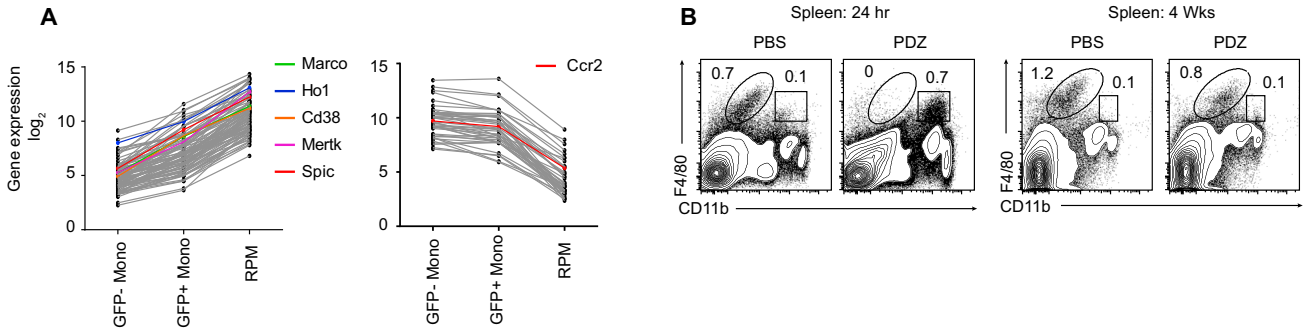

Figure S7. Pre-RPMs Are Intermediates between Monocytes and RPM, Related to Figure 4

(A) Splenocytes from *Spic*$^{igfp/+}$ mice were stained for F4/80, CD11b, and Ly6C and sorted for *Spic*-EGFP^{-} monocytes (CD11b^{hi}Ly6C^{+} *Spic*-EGFP^{-}), pre-RPM (CD11b^{hi}Ly6C^{+} *Spic*-EGFP^{+}), and RPM (F4/80^{hi}CD11b^{lo}*Spic*-EGFP ^{hi}). RNA was extracted from the sorted cells and gene expression analysis carried out using the Affymetrix expression platform. Gene expression values were normalized and analyzed using ArrayStar 4. Genes with $\geq$20-fold difference between EGFP^{-} monocytes and RPM were selected. Shown are the expression levels (y axis) of the selected genes (x axis) in the three sorted populations.

(B) WT mice were treated once with 200 μl of PBS or PDZ (2 mg in PBS). Splenocytes were harvested after one day (left) or 4 weeks later (right). Harvested splenocytes were stained with F4/80 and CD11b. Shown is the flow cytometry plots with the indicated markers. Numbers represent percentage of cells within indicated gate.